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APPLICATIONS OF IMMUNOFLUORESCENCE IN PUBLIC HEALTH VIROLOGY[1]

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INTRODUCTION

The demonstration by Coons and associates (5, 6) of fluorescent-antibody (FA) techniques as a means for the direct observation of antigens or antigen-antibody reactions presented the public health laboratory with a new tool for the development of rapid diagnostic methods for infectious diseases. This specific staining procedure was first utilized in the diagnosis of influenza (16) and was subsequently appli-d to other viral diseases.

The observation of the spatial localization of antigen within infected cells, afforded by immunofluorescence, lends itself to the routine diagnosis of diseases in which such localization occurs. This method is particularly useful when the cytoplasmic localization is characteristic, as in the inclusion bodies of rabies, or when the antigen may be chiefly of intranuclear concentration, as in cell cultures of simian viruses. In addition, the immunological specificity of the FA technique, with its freedom from useful but arbitrary tinctorial or morphological criteria, often results in greater sensitivity and reliability than the conventional staining methods employed for viral antigens. Moreover, variations in the design of the tests make possible the detection of specific antibodies, as well as antigens, in a variety of infectious processes. Finally, the speed, economy, and other advantages of the FA technique tend to outweigh certain disadvantages inherent in this test. Many of the technical limitations imposed by factors producing nonspecific staining, as pointed out by previous workers (3, 4, 24), are gradually being overcome, and the FA technique is gaining wider acceptance.

As with other immunological reactions, a maximum of specific potency and a minimum of heterogeneity in the interaction of antigen and antibody are highly desirable. Antisera are, therefore, selected with special regard to their virus-neutralizing titers. When a test requires cells containing virus, either as an unknown or as a "positive" control, the infected cells are selected and fixed, usually in the earlier stages of the infectious cycle. This is accomplished with fair accuracy in tissue-culture systems where observations of viral cytopathology indicate periods of virus release. In the case of rabies, the "positive" controls come either from the brains of infected animals obtained in the field or from mice inoculated in the laboratory with "street" virus.

DIRECT AND INDIRECT METHODS

Two methods are available for employing immunofluorescence. Antibodies against specific antigens are labeled in the so-called direct method (5, 6), or unlabeled antisera are placed in contact with antigen and then used with labeled antibodies against the particular animal species from which the specific serum was obtained (28). This indirect or "sandwich" technique permits the use of many sera from one animal species against different antigens with a single conjugate against the specific animal serum, in contrast to the use of a separate conjugate for each antigen.

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The advantage of the direct method is the elimination of the intervening antiserum, which is a potential source of nonspecific staining, particularly with human sera which frequently contain heterogeneous antibodies.

NONSPECIFIC FLUORESCENCE

A major problem peculiar to immunofluorescence is the reduction of nonspecific or background staining. This is particularly critical in cell-free diagnostic smears, because scattered viral antigen may be obscured by debris that has indiscriminately trapped the conjugate.

Nonspecific staining may result from the heterogeneity of the test preparation or of the antibodies used for the conjugate. Often this indiscriminate dye deposition results from free fluorescein present after labeling or after storage.

Various methods have been used to remove unbound fluorescein. They include prolonged dialysis at 4 C (6), diethylaminoethyl (DEAE) cellulose column fractionation (21, 26), charcoal adsorption (9), gel filtration (14, 10), and gel filtration followed by DEAE cellulose fractionation (23).

Purified conjugates usually require additional treatment prior to use. The most prevalent method has been adsorption with acetone-precipitated liver powder of mice or other species (6). Other methods involve tissue powder columns (17), acetone treatment after gel filtration (13), or tissue powder adsorption after gel filtration of undialyzed conjugate (15).

The method selected to reduce background or nonspecific staining may be determined by the needs and conditions of the individual laboratory. In our laboratory, a rapid method suitable for small volumes (1.0 ml) of labeled globulin was sought, to permit the processing and utilization of the conjugate the same day that a specimen arrived.

A method meeting these requirements, now used in our laboratory, combines dialysis after labeling followed by storage at −70 C with tissue powder adsorption and gel filtration immediately before use. Repeated tests have shown that this sequence will provide maximal positive and minimal nonspecific staining. The details of this technique will be published elsewhere.

In addition to the appropriate controls, coding of both control and test preparations is employed to insure objective evaluations. This method is particularly useful when background fluorescence is intensified by debris or when slight differences in brightness serve to indicate end points of antibody and antigen levels.

There are three cases in diagnostic virology which present the public health laboratory with dramatic problems. These are patients bitten by animals suspected of having rabies, cases of suspected smallpox (which invariably require differentiation from vaccinia, varicella, or herpes), and, more recently, the laboratory confirmation of rubella in a pregnant female. Each such specimen is accompanied by excitement, a sense of urgency, and the ever-present demand for a rapid and reliable presumptive report. The results of a slower and more sophisticated corroborative study may follow a little later. Immunofluorescence appears to be admirably suited to these situations, and attempts to apply and further perfect this technique are being made in our laboratories as well as in others.

RABIES

A rapid presumptive diagnosis for rabies based on aniline dye staining of Negri bodies has long been available. This method is still of great value, but its reliability depends upon an experienced individual. The FA method for rabies enjoys certain advantages over conventional stains. Since well-formed Negri bodies are not required, antigen in the form of small granules or deformed Negri bodies may be more easily discerned (11) by individuals lacking the long experience required in the classical method. Moreover, in certain preparations, such as smears of fixed rabies virus, which appear negative by conventional staining, the viral antigen is clearly and definitely observed with the FA technique (Fig. 1A and B). Another advantage of immunofluorescence is the demonstration of rabies virus in salivary glands, where, with conventional stains, virus particles are usually not demonstrable (12).

SMALLPOX

In New York City, as in other centers of travel, there is the constant threat of the introduction of variola by transients who arrive these days in only a few hours' time from endemic areas. When smallpox is suspected, a quick and accurate diagnosis is essential to prevent the undesirable detention of incorrectly diagnosed cases and

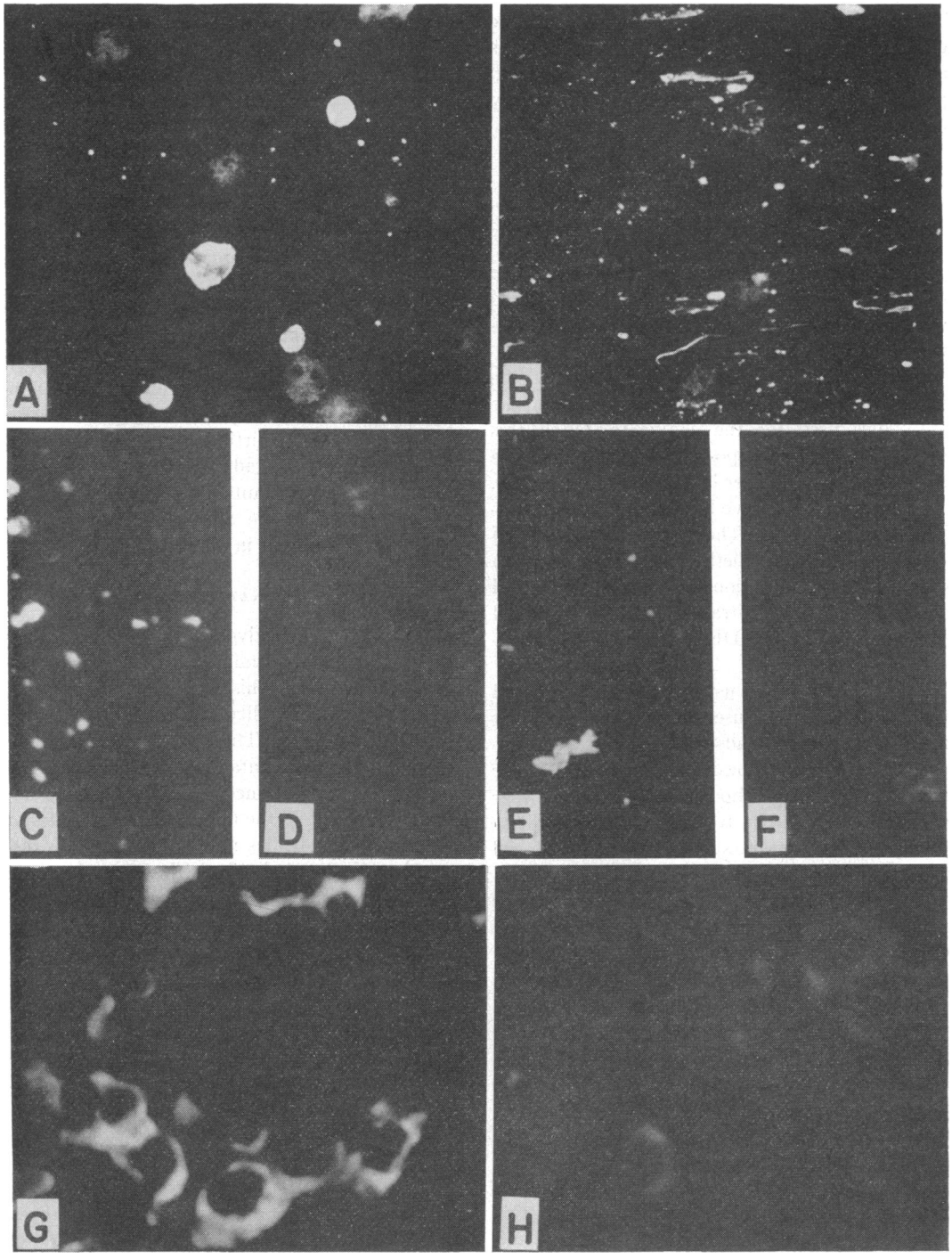

FIG. 1

their contacts. Rapid diagnosis may also minimize the possibility of costly, panicky, and usually unnecessary mass inoculations.

Although the elementary bodies of variola (and vaccinia) may be stained by conventional dyes and seen in cells scraped from the base of vesicular lesions, much experience in examining such preparations is required. The dearth of positive material provides little opportunity for the acquisition of this experience. To demonstrate virus in skin lesions, the laboratory worker has, therefore, resorted to the inoculation of material into the chorioallantoic membrane of the embryonated egg. This requires at least 2 days, and frequently one or two additional subpassages may be necessary. Complement-fixation tests for the detection of antigen in skin lesions is often difficult and may take 24 hr to complete. The demonstration of a rise in antibody titer in the patient's convalescent serum requires 6 to 10 days. In contrast, the demonstration of specific immunofluorescence in the patient's vesicular or pustular material can be completed within 4 hr after the skin lesion specimens arrive in the laboratory. Furthermore, dubious lesions appearing on the chorioallantoic membranes after inoculation may be readily identified by this technique (15, 17).

As with other immunological reactions, the FA method will not differentiate variola from vaccinia. Thus, the biological characteristics of the virus must subsequently be determined, and the clinical and epidemiological factors must be considered before a final decision is made by the physician or health officer who is responsible for the diagnosis.

TABLE 1. *Fluorescence reaction by indirect staining of human vesicle fluid**

Serum dilution	Unlabeled human serum					
	Patient's serum	Varicella positive	Herpes simplex positive	Vaccinia positive	Vaccinia negative	No serum
Undiluted	++++	++++	+/−	+/−	0	0
1:2	++++	++++	+/−	+/−	0	0
1:4	++++	++++	+/−	+/−	0	0

* Antigen: vesicle fluid from suspected generalized vaccinia (proved to be varicella). Conjugate: goat antihuman globulin labeled with fluorescein isothiocyanate. Reaction grading: 0 = no evidence of fluorescence; +/− = traces of dull-green fluorescence; ++++ = profuse bright yellow-green fluorescence.

VARICELLA AND OTHER VESICULAR ERUPTIONS

By far the most frequent condition encountered in smallpox "scares" in this country (and elsewhere where this disease is rare) is "atypical" chickenpox. Less frequently, herpes zoster and herpes simplex are involved in the differential diagnosis. Until recently, varicella and herpes zoster were "ruled out" upon the failure of growth of any agent in the chick embryo inocu-

FIG. 1A. *Impression smear of a dog brain infected with street virus, stained with fluorescent antibody. Note the large well-formed and brightly fluorescent Negri bodies. 620×.*

FIG. 1B. *Impression smear of a mouse brain infected with fixed rabies virus, stained with fluorescent antibody. Here the antigen is seen as discrete, brightly fluorescent bodies but smaller and more irregular than the Negri bodies observed in street virus preparations. 620×.*

FIG. 1C. *Smear of vesicle fluid from a patient with varicella, treated with varicella-positive human serum and stained with labeled antihuman globulin. Stained varicella antigen is observed. 250×.*

FIG. 1D. *Smear from the same specimen as in 1C, but treated with human antiherpes simplex serum and stained as above. No specific staining is seen. 250×.*

FIG. 1E. *Smear from same specimen as in 1C, treated with patient's convalescent serum and stained as above. This smear resembles that of 1C. 250×.*

FIG. 1F. *Smear from same specimen as in 1C, treated with human antivaccinia serum. No staining is noted after labeled antihuman globulin is applied. 250×.*

FIG. 1G. *Smear from African green primary kidney tissue culture infected with rubella virus, treated with rubella human convalescent serum and stained with labeled antihuman globulin. Note the brightly fluorescent cytoplasm in contrast to the nucleus. 250×.*

FIG. 1H. *Smear from same infected cells as in 1G, treated with normal rabbit serum and stained as above Note the absence of fluorescence. 250×.*

lated with vesicular material. Since the successful cultivation of these viruses, varicella and herpes zoster have been shown to be either similar or identical viruses by conventional immunological methods as well as by the FA method. Fluorescein-labeled antibody from either disease reacts with antigen in tissue cultures infected with virus obtained from patients with varicella or herpes zoster (28, 1). Excellent correlation exists between the isolation of virus from herpes simplex lesions and its demonstration by immunofluorescent staining (2). It is now possible, therefore, to identify any of the known viruses that may be present in vesicular eruptions.

An example of the application of this technique in a recent case of suspected generalized vaccinia that proved to be varicella is illustrated in Table 1 and Fig. 1C, D, E, and F. In general, smears from vesicle fluid, pustules, or scabs from patients with varicella are tested with human antiserum and antihuman serum conjugates by the indirect method. In the event vaccinia or herpes simplex is suspected, the greater abundance of labeled antibody permits the use of both direct and indirect methods. The indirect method is useful also in measuring antibody combining levels in a variety of diseases, particularly where the neutralization test or other serological tests may be cumbersome. This has been demonstrated with nonviral antigens (7, 27, 8, 19) as well as with enteroviruses (22) and rabies (22, 25).

TABLE 2. *Fluorescence in African green monkey primary kidney cultures infected with rubella virus**

Time after inoculation	Cells	Serum†	Serum dilution		
			1:4	1:8	1:16
days					
2	Uninfected	Negative	+/−	+/−	+/−
		Positive	+/−	+/−	+/−
	Infected	Negative	+/−	+/−	+
		Positive	+	+	+/−
		Normal rabbit	+/−	+/−	+
3	Infected	Negative	+	+	+/−
		Positive	++	++	+
8	Infected	Negative	+++	++	+
		Positive	++++	++++	++
10	Uninfected	Negative	+/−	+	+/−
		Positive	+/−	+/−	+/−
	Infected	Negative	++	+	+/−
		Positive	+++	++	++
		Normal rabbit	+	+/−	+/−

* Conjugate: goat antihuman globulin. Reaction grading: +/− = dull-green cytoplasm; + = bright-green cytoplasm, rare yellow-green; ++ = many cells with bright-yellow-green cytoplasm; +++ = about 50% cells with bright-yellow-green cytoplasm; ++++ = most cells with bright-yellow-green cytoplasm.

† Negative = human serum <1:4 by neutralization test; positive = human serum >1:4 by neutralization test.

Economy and speed are effected by placing six to eight dilutions of the serum to be tested on one standard microscope slide and treating these with antigen, conjugate, and other washing procedures simultaneously.

RUBELLA

We have applied such a technique for the detection of rubella antibodies. Previous attempts to demonstrate rubella virus by immunofluorescence in tissue cultures of chick embryo and primary human cells or cell lines of human origin have been unsuccessful (29), but we have

repeatedly observed specific staining of rubella virus in the cytoplasm of rubella-infected primary African green monkey (*Cercopithicus aethiops*) kidney cells (Fig. 1G). Table 2 shows results of a typical experiment in which staining of rubella virus is demonstrated. After this experiment, 60 serum specimens which were previously tested for rubella antibodies by the currently employed serum neutralization interference test (18) were retested with the FA technique (Table 3).

These preliminary results indicate agreement in about 80% of the specimens compared by the two methods. The reason for divergent results in the remaining 20% of the specimens is, as yet, not clear. Testing for unknown antibodies by FA requires maximal uniformity in the amount of

TABLE 3. *Comparison of rubella antibody results obtained by neutralization and immunofluorescence techniques*

Interference test titers	Immunofluorescence titers	
	Less than 1:4	Greater than 1:4
Less than 1:4		
15 samples	5 (33%)	10 (67%)
Greater than 1:4		
45 samples	8 (18%)	37 (82%)

known antigen. A slowly growing virus such as rubella (18, 29) may not readily provide a high percentage of maximally infected cells. To meet this need, experiments are in progress for obtaining maximal infectivity of the tissue-culture monolayer.

Clarification of the factors responsible for the discrepancies, and improvement of the accuracy of the test, should lead eventually to the replacement of the relatively complex neutralization test by the simpler FA procedure.

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